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Substrate specificity of angular dioxygenase from carbazole-degrading bacterium *Neptuniibacter* sp. strain CAR-SF

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ABSTRACT

Carbazole-degrading bacteria have been shown to have broad substrate specificity towards various contaminants. Carbazole 1,9a-dioxygenase (CARDO) from *Neptuniibacter* sp. strain CAR-SF composed of terminal oxygenase component CarAa, ferredoxin component CarAc, and ferredoxinreductase component CarAd.Expression vector encoding carbazole 1,9a-dioxygenase (CARDO) from *Neptuniibacter* sp. strain CAR-SF CARDO, pETCARA1 was constructed and dioxygenase activity was assessed by monitoring the blue-indigo production in Luria broth media and SDS-PAGE. Gas chromatography-mass spectrometry analysis revealed the angular dioxygenation of dibenzofuran at angular position adjacent to oxygen atom to yield 2,2',3-Trihydroxybiphenyl. CARDO also demonstrated activity towards dibenzothiophene and fluorene by converting the substrates into monooxygenation products, dibenzothiophene-5-oxide and 9-Fluorenone respectively. Cis-dihydrodiols and monohydroxylated products were also seen in the biotransformation of naphthalene, biphenyl and fluoranthene. These diverse oxygenations illustrated by CARDO revealed the broad versatility in its action on polyaromatic compounds and thus will make it as an excellent tool for bioremediation application.

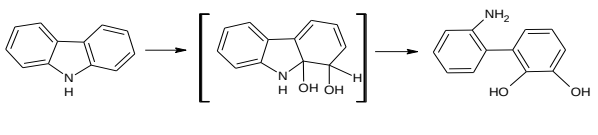
Keywords: Carbazole 1,9a-dioxygenase, angular dioxygenase, substrate specificity, carbazole

INTRODUCTION

Polycyclic and heterocyclic aromatic compounds are well known environmental pollutants that result largely from the incomplete combustion of coal, oil, petrol and wood. The fate of polyaromatic compound and its derivatives in nature is of great environmental concern due to their carcinogenic properties and also to be recalcitrant molecules. Based on the data deposited by Agency for Toxic Substances and Disease Registry (ATSDR), 17 polycyclic aromatic hydrocarbons (PAHs) have been identified as priority substances that pose a significant danger to public health. Their carcinogenic properties therefore call for a great attention in studying such chemicals and targeting their degradation pathways in order to detoxify and mineralize these ubiquitous environmental contaminants.

PAH dioxygenases are well known to have broad substrate specificities and perform various oxidation reactions, including *cis* dihydroxylation, mono- and di-oxygenation. In the field of bioremediation, it is imperative to have pollutant-degrading bacteria or enzymes with versatile oxidation activities since it is more often that bacteriawould be exposed to more than one organic compound in natural environment. Thus, angular dioxygenase is the most favourable dioxygenase as it can participate in various oxidation reactions. Besides, the initial oxidation to break the chemically stable aromatic ring is the most critical point in the degradation pathway of aromatic compounds in which the intermediate would become more accessible for degradation by other enzymes.

Previously, carbazole-degrading genes governing the angular attack on carbazole have been reported by Nagashima*et al.* from marine bacterium *Neptuniibacter* sp. strain CAR-SF. The terminal dioxygenase enzyme, known as carbazole 1,9a-dioxygenase (CARDO) composed of terminal oxygenase (CarAa), ferredoxin (CarAc) and ferredoxinreductase (CarAd). CARDO catalyzes the angular dioxygenation of carbazole to give the unstable dihydroxylated intermediate that will be converted to 2'-aminobiphenyl-2,3-diol (Fig. 1). Concerning that there is still a lack of knowledge on the CARDO from marine bacteria, it willbe quite interesting to investigate the similarities and at the same time to compare the substrate recognition by CARDO from terrestrial bacterium CA10 and marine bacterium CAR-SF. In this paper, we obtain the information on the substrate recognition of CARDO from marine bacterium *Neptuniibacter* sp. CAR-SF and identified theproducts generated by $CARDO_{CARSF}$from various aromatic compounds. This is the first report on the CARDO enzyme governing the angular attack from a marine bacterium.



Carbazole (CAR) 2'-Aminobiphenyl-2,3-diol

Fig.1.Conversion of CAR catalyzed by CARDO from *Neptuniibacter* sp. strain CAR-SF. The structure shown in bracket is an unstable intermediate that has not been characterized

MATERIALS AND METHODS

Bacterial strains and plasmids.*E.coli* strains XL1-Blue and BL21(DE3) were used as host strains for cloning and gene expression analysis, respectively. Cells were grown on Luria-Bertani (LB) broth supplemented with kanamycin (50 ug/ul) at 37°C. For plate cultures, LB medium solidified with 1.5% (wt/v) agar was used. pET41(a+) was used as an expression vector under the control of the T7 promoter-driven system in *E.coli*. Bacterial strains and plasmids used in this study were summarized in Table 1.

DNA manipulation. The carAaAcAd genes were synthesized by Genewiz, Inc. (South Plainfield, NJ). Nucleotide sequences used in this study were registered under GeneBank accession number AB123456 and AB123456. The genes were cloned into pUC57-kan and the resulting plasmid was designated as pUCARA1. Plasmids DNA were prepared using PureYield™ Plasmid Miniprep (Promega, Madison) as described by the manufacturer.

Construction of expression plasmid. To express the CARDO genes under control of the T7 promoter-driven system in *E.coli*, *carAaAcAd*was amplified from pUCARA1 by PCR amplifications using specific primers for carA gene. The forward primer (5'-ATCCATGGTAAGCATGGCTAATGTATCAGAAG-3') was designed to add an *NcoI* site upstream of the initial codon. The reverse primer (5'-GCGC*GAATTC*TTAGAAAAATGCGTCAA-3') was designed to add an *EcoRI* site downstream of the stop codon. The resultant PCR amplicons were double-digested at the introduced restriction sites before ligation into the corresponding sites of plasmid vector pET41a vector (Novagen). The resulting recombinant plasmid, pETCARA1 was transformed into *E.coli*BL21 (DE3) cells according to the method of Inuoe.

Dioxygenase assay and preparation of crude cell extract. Oxygenase activity was examined by monitoring the blue-indigo production as a result of CARDO genes expression. The recombinant were grown on LB medium and isopropyl β-D-1-thiogalactopyranoside (IPTG) was supplied at 1mM when the absorbance at 600nm reached 0.5 . The crude cell extract was prepared using the BugBuster protein extraction reagent and standard protocol was followed (Novagen). SDS PAGE analyses were carried out according to the method of Laemmli and detection of proteins was performed by staining with Coomassie brilliant blue R-250.

Biotransformation of CAR analogues, PAHs and biphenyl. Resting cells of *E.coli* strains were prepared as described previously. Substrates were dissolved in dimethyl sulfoxide (10mg/ml) and then 50ul of this solution was added to 5-ml cell suspension in CNF buffer. After 20 h of incubation at 30°C, the mixtures were extracted with an equal volume of ethyl acetate after acidification to pH 2 with 1N HCl and dried over anhydrous sodium sulphate. The extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) as described previously. In some cases, extracts were derivatized at 70°C for 20 minutes with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) to assist in identification of acidic metabolites.

GCMS analysis and identification of metabolites. The GCMS analysis was performed using a Shimadzu system with GC-MS-QP2010 quadrapole mass spectrometer equipped with a splitless injector as described previously. To quantify the substrate remaining and the products formed, we compared the peak area of the total ion current (TIC) of the compounds extracted from the reaction mixture containing *E.coli* cells harbouring pETCARA1 with that of the substrate extracted from the reaction mixture containing *E.coli* cells harbouring pET41a(+)1 [4, 6]. The conversion ratio was calculated based on three independent biotransformation experiments. Chemical structures of metabolites were suggested on the basis of their mass fragmentation patterns, an instrument library search and previously reported literature data.

Table.1.Bacterial strains and plasmids used in this study

Bacterial strains	Relevant characteristics	Source or reference
***E.coli* XL1-Blue**	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lac*IqZΔ*M15* Tn*10* (Tetr)]	Agilent
***E.coli* BL21 (DE3)**	*E. coli* B F- *dcmompThsdS*(rB- mB-) *gal* λ(DE3)	BioLabs, New Zealand
Plasmids		
pUCARA1	Kan^r, pUC57 with *carAa* gene of CAR-SF and *carAcAd*genes of CA10	This study
pETCARA1	Kan^r, pET41a(+) with 2.5-kb *carAaAcAd* fragments from pUCARA1	This study

RESULTS AND DISCUSSION

Functional expression of carAaAcAd in *E.coli*: Oxygenase activity of the*E.coli*cells was confirmed as the formation of the blue pigment was sought in the culture medium as the result of the combined activities of tryptophanase and dioxygenase. SDS-PAGE analyses revealed the predicted molecular masses of carAa and carAd gene products at 44 and 36 kDa respectively. As for carAc, the gene product could not clearly be detected in *E. coli*

harboring pETCARA1. This was most probably due to weak expression as in the case of the carAc gene product in Pseudomonas sp. CA10. The weak expression by carAc also suggested that there would be a rather indefinite association between the dioxygenase components. However, the amount of carAc expressed in *E. coli* might be sufficient to transport electrons to the terminal component.

Substrate range of carAaAcd: The substrate specificity of $CARDO_{CARSF}$ was investigated in the resting cell reaction and the results of the physical properties of the compounds formed were summarized in Table II. The substrate specificity of $CARDO_{CARSF}$ was compared with those of the well-studied CARDO from terrestrial bacterium *Pseudomonas resinovorans* CA10 and angulardioxygenasesdibenzofuran 4,4a-dioxygenase (DFDO) from *Terrabacter* sp. strain DBF63 and fluorenedioxygenase complex (FlnA1-FlnA2) from Sphingomonas sp. strain LB126 as shown in Table III. In the biotransformation experiment, the major product detected from oxidation of carbazole (CAR) by CARDO was 2'-Aminobiphenyl-2,3-diol (98%), and this was identical to that reported earlier by Nagashima*et al*.. $CARDO_{CARSF}$ catalyzes the angular oxidation of CAR and attacks at the angular position adjacent to the nitrogen atom (Fig. 3A). Traces amount of monohydroxycarbazole was also detected in the incubation mixture suggesting that $CARDO_{CARSF}$ can also catalyze the cis-dihydroxylation of PAHs. When compared to other PAH dioxygenases, CARDO is the only dioxygenase that can convert CAR into angular dioxygenation product, 2'-Aminobiphenyl-2,3-diol.

Three heteroatomic analogs of CAR, i.e., dibenzofuran (DBF), dibenzothiophene (DBT) and fluorene (FLO) were tested as substrates for angular oxidation in the biotransformation experiment. DBF was converted into a compound having a molecular peak ion at *m/z*418. Together with GC-MS analysis and literature data, this compound was identified as 2,2',3-Trihydroxybiphenyl. This metabolite was previously identified as metabolic intermediate of dibenzofuran degradation by DFDO and $CARDO_{CA10}$. The formation of the angular product signified that DBF could somehow approach the reaction center of $CARDO_{CARSF}$ similar to CAR in which the angular attack occurred at the angular position adjacent to oxygen atom (Fig. 3B). In addition to dioxygenation, $CARDO_{CARSF}$ could also converted significant amount of DBT in the resting cell reaction, transforming over 44% of DBT into monooxygenation product, dibenzothiophene-5-oxide (Fig. 3C). Similar sulfoxidation of DBT was seen with previously well-studied $CARDO_{CA10.}$ Previously, transformation of DBT to another sulfoxidation product, dibenzothiophenesulfone was seen with both angular dioxygenases DFDO and FlnA1-FlnA2 [17, 18]. $CARDO_{CARSF}$could also transformed DBT via lateral dioxygenation, evident by the production of monihydroxydibenzothiophene in the resting cell reaction. This result demonstrated that DBT can approach the reaction center of $CARDO_{CARSF}$ in at least two different ways.

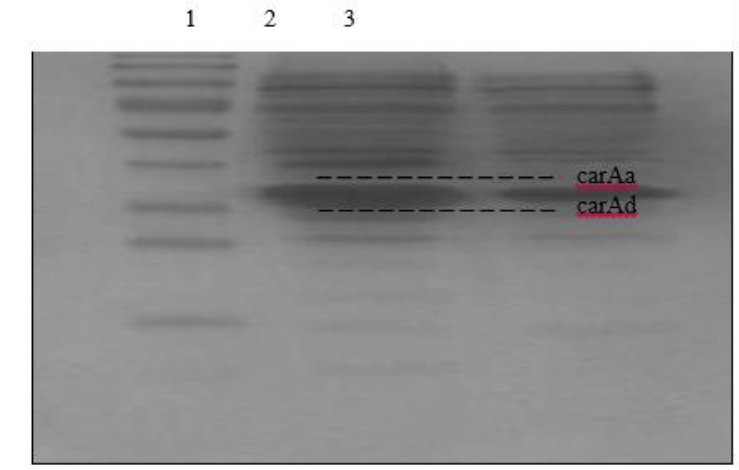


Fig.2.Detection of the products carAa and carAd. Total cellular proteins of E.coli strains were analysed by SDS-PAGE. Lane 1, molecular mass standards of 170, 130, 95, 72, 56, 43, 34, 26, 17 and 11 kDa (top to bottom); Lane 2, BL21 (pETCARA1); Lane 3, BL21 (pET41a(+))

Considering the fact that $CARDO_{CARSF}$ was able to perform angular dioxygenation of DBF and sulfoxidation of DBT, monooxygenation of FLO was speculated. In this study, $CARDO_{CARSF}$ converted FLO to two major products, 9-Fluorenone and 9-Hydroxyfluorene (Fig. 3D). In contrast, 9-Fluorenone was not seen with the reaction of FLO with $CARDO_{CA10}$. 9-Fluorenone is thought to be formed as anoxidation product of a nonspecific dehydrogenase from *E.coli*, which oxidized 9-Hydroxyfluorene to 9-Fluorenone. This was confirmed when formation of 9-Fluorenone was detected during the incubation of 9-Hydroxyfluorene with the control strain lacking the terminal dioxygenase gene construct. Formation of 9-Hydroxyfluorene suggested that $CARDO_{CARSF}$similar with $CARDO_{CA10,}$ is also able to catalyze the monooxygenation of benzylicmethylenic group of FLO since no such

reaction was seen with DFDO and FlnA1-FlnA2. The manner of oxygenation catalysed by $CARDO_{CARSF}$as seenwith the CAR analogs proved that CARDO mainly attacks at the same side in which the heteroatoms located, as seen with DBF, DBT and FLO.

The ability of $CARDO_{CARSF}$ to oxygenate a higher molecular weight compound was tested onfluoranthene (FLT). Only 4% of the substrate was converted to 7-Hydroxyfluoranthene assuming that the substrate binding site of CARDO was less efficient towards 4-membered ring compound. The same metabolite was observed with $CARDO_{CA10}$. 7-Hydroxyfluoranthene was believed to be produced as the result of dehydration of *cis*-dihydrodiols as the metabolites extraction was done in acidic condition. Thus, the presence of *cis*-7,8-dihydroxy-7,8-dihydrofluoranthene (Fig. 3E) was presumed. Besides, *cis*-Dihydroxylation at the 7 and 8 positions of FLT was seen as the initial oxygenation product of FLT with *Mycobacterium* sp. strain PYR-1. FlnA1-FlnA1 is the only PAHdioxygenase that can efficiently catalyze the *cis*-Dihydroxylation of FLT where 100% conversion was achieved after 48 hours of incubation.

Incubation with naphthalene (NAP) produced the expected metabolites, cis-dihydrodiols (Fig 3F). However in this study, *cis*-1,2-dihydroxy-1,2-dihydrobiphenyl was not seen as the oxygenation product of biphenyl (BPH). Only 2-Hydroxybiphenyl was detected as the major product and this monohydroxylated product was presumed to be formed from the cis-dihydrodiols by nonenzymatic dehydration under acidic condition. However, $CARDO_{CARSF}$transformed BPH less efficiently where only less than 30% of BPH was converted into oxygenation products compared to 100% conversion of BPH by $CARDO_{CA10.}$ This substrate preference shown by $CARDO_{CARSF}$demonstrated that this enzyme is less specific towards BPH and the substrate recognition is more towards CAR analogs since it mainly attacks at the same side of heteroatoms as illustrated by reactions with DBF, DBT and FLO (Fig. 3B, 3C, 3D).

Table.2.Physical properties of the compounds formed during the biotransformation of heterocyclic aromatic compounds, PAHS and biphenyl by CARDO detected by GC-MS

Substrate[a] (% remaining[b])	GCMS Data[c]		Identification or possible structure[d]	Yield (%)[b]
	R_f(min)	Principle ions and relative abundance (% base peak)		
Carbazole (ND)	15.8	345 (M^+, 14), 330 (14), 255 (5), 239 (3), 73 (100)	2'-Aminobiphenyl-2,3-diol	97.9
	16.2	255 (M^+, 100), 239 (63), 224 (65), 209 (15), 180 (10)	Monohydroxycarbazole	2.1
Dibenzothiophene (55.9)	17.1	200 (M^+, 4), 184 (100), 171 (3), 152 (14), 139 (27)	Dibenzothiophene-5-oxide	44.1
	15.7	272 (M^+, 82), 257 (51), 241 (100), 121 (26), 73 (62)	Monohydroxydibenzothiophene	Tr
Dibenzofuran (55.0)	15.3	418 (M^+, 9), 403 (2), 315 (19), 73 (100)	2,2',3-Trihydroxybiphenyl	45.0
Naphthalene (ND)	13.5	306 (M^+, 12), 276 (10), 206 (48), 191 (100), 147 (20)	*cis*-1,2-Dihydroxy-1,2-dihydronaphthalene	91.0
	12.2	216 (M^+, 100), 201 (90), 185 (55), 145 (5), 73 (65)	1-Naphthol	9.0
Fluorene (68.8)	14.1	180 (M^+, 100), 152 (65), 125 (25), 76 (55)	9-Fluorenone	18.7
	14.3	254 (M^+, 50), 239 (15), 223 (2), 178 (3), 165 (100)	Monohydroxyfluorene	10.3
	15.1	254 (M^+, 100), 239 (45), 223 (90), 178 (10), 165 (17)	2-Hydroxyfluorene	1.0
	15.2	182 (M^+, 100), 165 (36), 152 (65), 126 (13), 115 (10)	9-Hydroxyfluorene	1.2
Fluoranthene (96.0)	17.1	290 (M^+, 100), 275 (50), 259 (75), 244 (9), 215 (16)	7-Hydroxyfluoranthene	4.0
Biphenyl (72.0)	12.1	170 (M^+, 90), 141 (55), 139 (18), 115 (100), 102 (7)	2-Hydroxybiphenyl	25.0
	12.6	242 (M^+, 55), 227 (75), 211 (100), 165 (7), 152 (8)	4-Hydroxybiphenyl	2.0
	13.7	242 (M^+, 70), 227 (100), 211 (75), 165 (5), 152 (14)	3-Hydroxybiphenyl	1.1

[a]The starting concentration of substrates used was 100 ug/ml.

[b]To quantify the yield of remaining substrates, the peak area for the total ion current of each compound extracted from the incubationmixture prepared with *E.coli* cells harbouring pETCARA1 with the substrates extracted from the incubation mixture of*E.coli* cells harbouring pET41a+. The yield was calculated from transformation ratio of three independent experiments.

[c]Products were quantified by GCMS analyses. GC-MS data for all TMS derivative.

[d]Products were identified by comparisons of their mass spectra with data reported in the literature and the NIST library

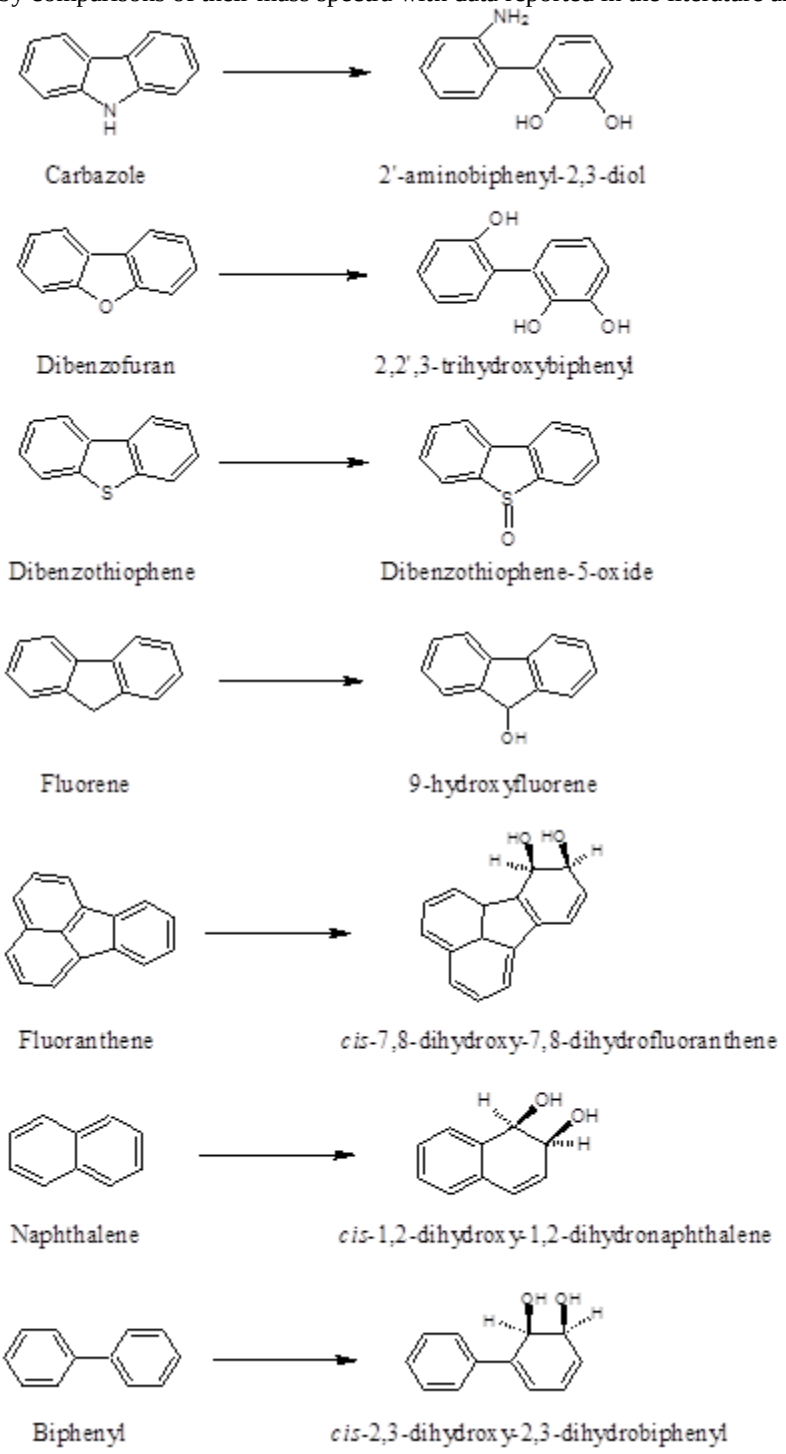


Fig.3Oxidation reaction catalysed by CARDO from *Neptuniibacter* sp. strain CAR-SF identified by GC-MS for (A) carbazole, (B) dibenzofuran, (C) dibenzothiophene, (D) fluorene, (E) fluoranthene, (F) naphthalene, (G) biphenyl. Monohydroxylated compounds were also detected in the biotransformation experiments for all substrates (A) – (G). Absolute stereochemistry is not intended.

Table.3.Substrate specificity of $CARDO_{CARSF}$ and other angular dioxygenases

Substrate	Reaction[a]	Product	Transformability by[b]:			
			CARDO CARSF	CARDO CA10	DFDO	FLNA1-FLNA2
Carbazole	AD	2'-Aminobiphenyl-2,3-diol	+	+	-	-
	LD	Monohydroxycarbazole	+	-	-	+
Dibenzofuran	AD	2,2',3-Trihydroxybiphenyl	+	+	+	+
Dibenzothiophene	MO	Dibenzothiophenesulfoxide	+	+	+	+
Fluorene	MO	9-Fluorenone	+	-	-	-
	MO	9-Hydroxyfluorene	+	+	-	-
Fluoranthene	LD	7-Hydroxyfluoranthene	+	+	NT[b]	+
	LD	*cis*-2,3-dihydroxy-2,3-dihydrofluoranthene	-	+	NT	-
Biphenyl	LA	*cis*-1,2-dihydroxy-1,2-dihydrobiphenyl Dihydrodiols	ND[b]	+	+	+
	LA		+	+	+	+
Naphthalene	LA	*cis*-1,2-dihydroxy-1,2-dihydronaphthalene	+	+	+	+
	LA	1-Naphthol	+	+	+	+

[a]Oxygenation reaction was designated as angular dioxygenation (AD), lateral dioxygenation (LD) and monooxygenation(MO).

[b]The ability and inability to convert the substrate to each product are shown by '+' and '-' respectively. NT-not tested. ND-not detected.

CONCLUSION

The findings shown in this study indicate that the angular dioxygenase from marine bacterium CAR-SF is indeed unique that it showed broad versatility in its action on polyaromatic substrates and was most closely related to proteins involved in the angular attack on carbazole by terrestrial bacterium *Pseudomonas* sp. strain CA10. Although $CARDO_{CARSF}$also shares characteristics with both angular dioxygenases DFDO and also FlnA1-FlnA2, CARDO is still the only dioxygenase that can efficiently perform angular attack on CAR analogs, monooxygenation and *cis*-Dihydroxylation.Considering the fact that $CARDO_{CARSF}$ has broad substrate specificity, the transformantsharbouring pETCARA1 could be a useful tool for remediation of dioxin and dioxin-like compounds in a closed system and also for bioremediation of various aromatic-compound contaminated sites. The evaluation of the substrate specificity of the metacleavage dioxygenase (carB) is currently under way.

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